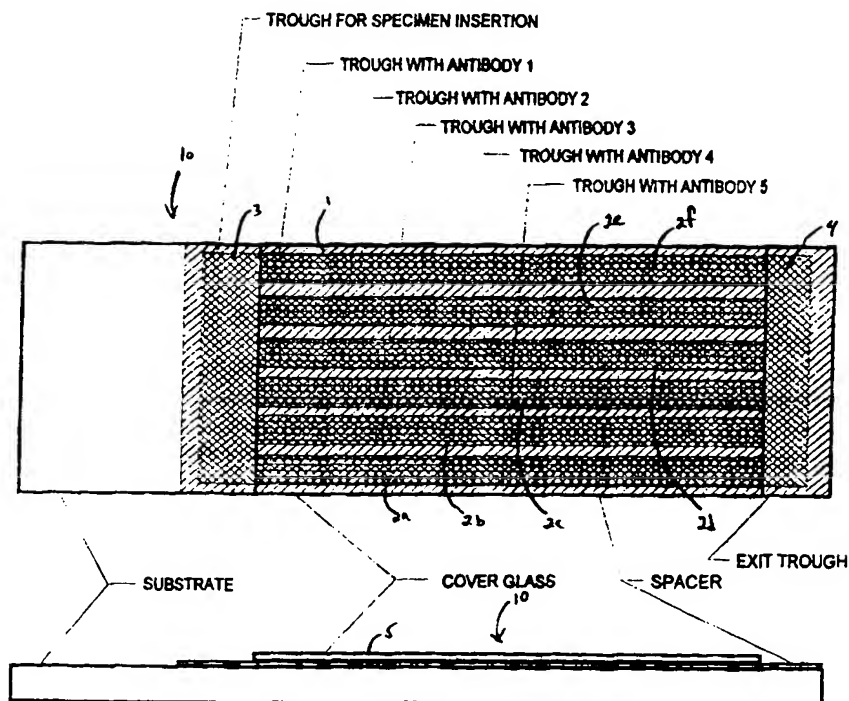


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>G01N 33/48, 21/00, 21/01, 21/03, 1/10, B01L 3/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/41863</b> <b>(43) International Publication Date:</b> 24 September 1998 (24.09.98)
<b>(21) International Application Number:</b> PCT/US97/03900 <b>(22) International Filing Date:</b> 14 March 1997 (14.03.97) <b>(71) Applicant:</b> COMPUCYTE CORP. [US/US]; 12 Emily Street, Cambridge, MA 02139-4517 (US). <b>(72) Inventor:</b> CLATCH, Richard, J.; 3823 Chester Drive, Glenview, IL 60025 (US). <b>(74) Agent:</b> NISSENBAUM, Israel; Graham & James LLP, 885 Third Avenue, New York, NY 10022 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>

**(54) Title:** MULTIPLE SIMULTANEOUS TESTING MEDIA**(57) Abstract**

A stationary testing media device containing a glass or plastic plate (10) and conveniently a microscope slide, wherein multiple simultaneous tests are conducted on a single multifurcated testing sample. The plate or slide (10) is partitioned into two or more testing zones (2a-2f) with each of the zones having been preferably pre-loaded with a stable test reactant. The testing zones (2a-2f) are physically separated from one another but are connected, with a fluid flow connection, to a single sample reservoir (3) which simultaneously feeds, such as by capillary action, each of the testing zones (2a-2f) with a test portion of the testing sample. The testing zones (2a-2f) retain the individual portions for a time sufficient to effect the individual zone reactions with pre-loaded reagents. An optional outflow reservoir (4) is utilized to capture excess sample flow and flushing fluids. Cell samples undergoing laser scanning cytometry are able to be simultaneously reacted, such as with varying fluorescent dyes, to provide a series of test profiles with a single sample and testing procedure.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NI	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**MULTIPLE SIMULTANEOUS TESTING MEDIA****FIELD OF THE INVENTION**

This invention relates to diagnostic testing procedures and testing vehicles such as glass or plastic plates and particularly to microscope slides used in laser scanning cytometry.

**BACKGROUND OF THE INVENTION**

Microscope diagnostic characterization of hematologic neoplasms is increasingly dependent on immunophenotypic analysis, often performed by flow cytometry. Immunophenotyping by flow cytometry provides objective data that is necessary for the subclassification of leukemia and lymphoma, and that can often definitely establish or exclude a diagnosis of malignancy in seemingly borderline cases. Immunophenotyping of peripheral blood leukocyte subsets is also an important laboratory indicator for patients infected with the human immunodeficiency virus (HIV) that causes the acquired immunodeficiency syndrome (AIDS).

Flow cytometers are used routinely to provide dual antibody profiles using patient blood and other specimens such as tissue biopsies. Each set of dual measurement requires a separate tube containing cells treated with two antibodies. Each tube is independently run on the flow cytometer and the results of all measurements are combined on a report. Although it is possible to measure as many as four different antibodies at the same time on each cell with a flow cytometer, this is technically difficult and expensive. Therefore, multiple aliquots of dual antibodies are more commonly utilized.

Drawbacks of flow cytometry include relatively large specimen size requirements and the inability to microscopically examine the cells being studied. Furthermore, in order to normally accomplish multi-parameter immunophenotyping (greater than four immunofluorescent antibodies per specimen), flow cytometric analysis requires that separate subsets of a single specimen be manually or robotically placed within the flow cytometer and analyzed separately.

Until now, alternatives to flow cytometry have been few, including only immunohistochemical methods that suffer from a lack of automation and objectivity, and that generally compromise the number of antigens that can be analyzed.

5 A device, referred to as a laser scanning cytometer, available from CompuCyte Corp. (under the trademark "LSC", as described in US Patent No. 5,072,382, the disclosure of which is incorporated herein by reference thereto), has been recently developed which provides an alternative to flow cytometric immunophenotyping, and  
10 offers several unique advantages. Similar to flow cytometry, laser scanning cytometry provides objective automated, multi-color analyses of hematologic cell suspensions, stained by using conventional immunofluorescent techniques. However, laser scanning cytometry also allows for the visual examination of  
15 individual cells both during and after analysis, and makes possible the analysis of very small diagnostic samples, such as those obtained via fine needle aspiration biopsy. This is true primarily because for laser scanning cytometry, the specimens are analyzed on a static surface (such as a glass microscope slide) rather than within a fluid stream as with flow cytometry. An  
20 added advantage and the principal idea behind the present invention is that for laser scanning cytometry true multi-parameter immunophenotyping of a single specimen (or even multiple specimens) is possible using only a single glass microscope slide or other vehicle to load the specimen into the laser scanning  
25 cytometer.

Preparation of a specimen for immunophenotypic analysis is performed by reacting aliquoted subsets of that specimen (typically constituting a suspension of purified hemopoietic or  
30 lymphoreticular cells) with various mixtures of fluorochrome-labelled antibodies. After this reaction, the aliquoted subsets are washed and analyzed individually via flow cytometry or laser scanning cytometry. Typically each aliquot is reacted simultaneously with a mixture of up to four different antibodies  
35 labelled with different fluorochromes. This is possible because of differences in the spectral characteristics of the fluorochromes utilized and because the instrumentation of flow

cytometer or the laser scanning cytometer is designed to simultaneously but discretely assay emitted light from such fluorochromes. Because of the cost of producing different antibodies labeled with various fluorochromes and the cost and complexity of instrumentation necessary for the assay, individual aliquoted subsets of a single specimen are usually simultaneously reacted with only two or three different antibodies. However, modern immunophenotypic analysis usually requires assessment of multiple (usually 5 to 15) different antigens, and it is often clinically necessary or desirable to assess a single particular antigen in the context of multiple different other antigens. Therefore, specimens are presently aliquoted and reacted with multiple mixtures of various fluorochrome-labelled antibodies to assess the expression of the corresponding antigens and their relationships with one another.

For testing of the multiple mixtures, at least three manufacturers sell slides with multiple compartments or devices which can create multiple compartments. These do not, however, provide means for generating an assay and they also require separate aliquot samples for each of the compartments or chambers.

#### SUMMARY OF THE INVENTION

It is an object of the present invention to provide a stationary testing media embodied in a testing plate made of glass or plastic and conveniently embodied in a microscope slide, wherein multiple simultaneous tests are conducted on a single self-multifurcated testing sample.

It is a further object of the present invention to provide such media for use with laser scanning cytometry devices.

These and other objects, features and advantages of the present invention will become more evident from the following discussion and drawings in which:

### SHORT DESCRIPTION OF THE DRAWINGS

Figure 1 is a top view of a multi-chambered slide of the present invention;

Figure 2 is a side view of the slide of Figure 1;

5 Figure 3 is a top view of a second embodiment of a multi-chambered slide of the present invention;

Figure 4 is a side view of the slide of Figure 3;

10 Figures 5a-g are multiple profile test results from a single sample, obtained by using the multi-chambered slide of Figures 1 and 2.

Figures 6a-e are test results obtained by using prior art individual test sample runs with single tube assays.

### DETAILED DESCRIPTION OF THE INVENTION

15 Generally the present invention comprises a multiple simultaneous testing media device and testing method, which utilizes the ability of a laser scanning cytometer to geographically locate and distinguish different regions of one or more specimens  
20 constituting a single analysis. The preferred embodiment of the device comprises a substrate such as a glass or plastic plate which is partitioned into two or more testing chamber zones with each of the zones having been pre-loaded with a stable test  
25 reactant. The testing zones are physically separated from one another but are connected, with a fluid flow connection, to a single sample reservoir which simultaneously feeds, such as by capillary action, each of the testing zones with a test portion of the testing sample. The testing zones retain the individual  
30 portions for a time sufficient to effect the individual zone reactions with pre-loaded reagents. An optional outflow reservoir is utilized to capture excess sample flow and flushing fluids. In less preferred embodiments, the individual test reactants are separately loaded into the testing chamber zones together with the

individual test portions samples or thereafter into the test portions, by means such as pipetting.

The present invention further comprises a method of conducting at least two simultaneous tests with a laser scanning cytometer, with a single test sample on a single glass or plastic plate such as a microscope slide, said method comprising the steps of:

a. providing the plate or slide with a test sample reservoir and at least two separated sample portion troughs in fluid connection with the reservoir;

b. providing each of the sample troughs with a stable reactant for a test sample, preferably prior to introduction of the test sample;

c. placing a fluid test sample in said reservoir wherein fluid connection means causes portions of the test sample to flow into each of the at least two separated sample portion troughs;

d. maintaining sufficient cells in each of the portions in said portion troughs for a time sufficient for the cells to react with the stable reactant within the individual portion troughs; and

e. removing excess reactant whereby test results can be meaningfully obtained by the laser scanning cytometer with minimized background noise.

Cell samples undergoing laser scanning cytometry are able to be simultaneously reacted, such as with various fluorescent dyes, to provide a series of test profiles with a single sample and testing procedure. The device comprises a loading vehicle for laser scanning cytometry in which different geographic regions of the loading vehicle correlate with different test conditions. With respect to immunophenotypic analysis, this provides for multiple simultaneous reactions of one or more samples with multiple mixtures of fluorochrome-labelled antibodies. The vehicle is then loaded into a laser scanning cytometer; and, by means of a single comprehensive analysis of the material on the loading vehicle, multiple reaction mixtures or "geographic aliquots" of one or more specimens are assayed automatically.

Various embodiments of the present invention, relating to

immunophenotypic analysis, can be broadly divided into two groups. One group relies on cells becoming affixed to the loading device whereas the other relies on the cells not becoming affixed.

Affixation of lymphoreticular cells to a glass or plastic surface within the loading device is largely a function of cell type, the physical characteristics of the surface, and the manner in which the cells and surface are brought together.

#### **EMBODIMENT OF SURFACE AFFIXED CELLS:**

Thus, in a preferred embodiment, cells become affixed to a surface within the loading device, which is essentially a modified glass or plastic plate or conveniently a microscope slide. The plate is divided into several geographic regions either horizontally or vertically. It is preferred to create regions oriented horizontally (x-axis) in the device, in order to take greatest advantage of the direction of movement of the stage of the laser scanning cytometer, but vertically (y-axis) oriented regions work equally well.

With a typical slide it is preferred to create up to six distinct regions, because this nicely correlates to different analytical groups necessary for complete immunophenotyping of leukemia or lymphoma specimens as hematopathology is practiced today. However, the absolute number of regions created is essentially arbitrary.

A simple, yet effective manner of creating the six horizontally (x-axis) oriented regions, as a demonstration of the effectiveness of the device, is by means of very thin (e.g., 1 mm) strips of polymeric based double-stick adhesive tape, essentially inert to standard test specimens. Two or three thicknesses of conventional Scotch® brand Double Stick Tape works exceptionally well. Since a glass microscope slide is approximately 25 mm in vertical (y axis) dimension, six regions, i.e., chamber zones, are created with each having an approximate width of 2.5 mm. The regions are formed with 10 mm length x 1 mm width strips of tape being placed on the glass slide, as region boundaries. The regions are covered with a 24 x 40 mm glass coverslip to create six geometrically separate open ended capillary gap chambers within a single loading device.



At one of the two open ends of the capillary gap chambers, a trough or specimen reservoir, is formed whereby fluids placed in the trough simultaneously enter all six chambers. In a preferred embodiment the loading device is unitarily molded out of plastic, or assembled from plastic and glass.

An advantage of the device of the present invention is that fluorochrome-labelled antibodies (singly, in pairs, or in triplets) can be preloaded into the chambers, with each chamber holding a different antibody combination. These antibodies are most advantageously preloaded prior to sale of the device, and may be either in a stable liquid or dried state. Antibodies predried within separate chambers prior to coverslipping have maintained their immunologic and fluorescent activity to a significant degree.

The method of using the device for immunophenotyping comprises initial loading of a single aliquot of a relatively small specimen (approximately 300  $\mu$ l containing approximately 200,000 cells) into the trough, which automatically fills all six chambers completely (approximately 50  $\mu$ l each). The suspension of cells mixes with the antibodies specific to each chamber and is allowed to react for approximately 30 minutes at room temperature. If untreated glass microscope slides are used to make the device, more than 90% of the cells will have settled and become affixed to the slide surface at the end of this time. Thereafter approximately 1 ml of saline wash solution is placed into the trough and either allowed to drain through the six chambers by gravity or by means of a fixed volume of absorbent material placed at the exit end of the chambers. If an unlimited amount of absorbent material is available, the chambers will however be unacceptably drained dry. With the aforementioned procedure, more than 90% of the cells remain affixed to the surface of the loading vehicle for subsequent immunophenotypic analysis.

#### **EMBODIMENT OF NON-SURFACED AFFIXED CELLS:**

Immunophenotypic analysis using a geographically partitioned loading device wherein the cells do not become affixed to a surface within the device has some advantages as compared with the

embodiment described above but, may also have greater disadvantages and is less preferred. The loading device for this embodiment is similarly created using a glass microscope slide, double stick tape, and a coverslip.

- 5 Glass slides treated for immunohistochemistry have an interesting property that lymphoreticular cells in general do not become affixed to them. As before, horizontally (x-axis) oriented capillary chambers are created using thin strips of two or three thicknesses of double stick tape. However, for this embodiment, 10 the tape is cut into shapes slightly more complex than simple strips, each capillary chamber is itself partitioned into two regions, one quite small (5  $\mu$ l volume) at the entrance end of the chamber and another relatively large (45  $\mu$ l volume) in the middle and at the exit end of the chamber. The two regions of each 15 chamber are connected by only a small gap through which cells and diluent can flow only if sufficient volume pressure is applied to the loading end of the chamber. The different antibodies may be preloaded into the 5  $\mu$ l regions of the chambers and remain in liquid or dry state as described above.
- 20 A trough (or reservoir) similar to that described above is utilized to automatically load specimens into each of the six chambers. However, in this embodiment, the immunologic reactions are occurring in only 5  $\mu$ l of volume and so the quantity of antibody necessary is far less (1/10th of that as described above) 25 and the specimen must be 10 times more concentrated but in 1/10th the volume. Thus, for example, 200,000 cells would be loaded in only 30  $\mu$ l of diluent. After the reactions are complete (after approximately 30 minutes at room temperature), the trough is filled with 270  $\mu$ l of diluent, which is sufficient to force the 30 cells from the 5  $\mu$ l region of each chamber into the larger 45  $\mu$ l region. Although not a true wash, this constitutes a 1/10 dilution of unbound antibody, which is sufficient to reduce background fluorescence levels to an acceptable level for analysis.
- 35 An advantage of this embodiment is that all reagents remain completely within the device and do not need to be drained or

absorbed from the exit end of the chambers. An additional advantage is the savings with respect to quantities of antibody used.

5 In order to obtain advantages of both embodiments, a device having two region chambers, as described is made with a non-sticky surface within the 5  $\mu$ l chamber and a sticky surface within the 45  $\mu$ l chamber.

10 In a further highly preferred embodiment (common to both the above enumerated cell type embodiments), the dimensions of the troughs or chambers are controlled to provide known volumes via known (or determinable) chamber depths, in order to ascertain characteristics of the separately tested and laser scanned samples in each of the chambers. The LSC™ laser scanning cytometer contains software which counts the various cells in each region  
15 of a scattergram as well as cells displayed on the scattergram and is able to provide the ratio of cells counted in a region to all cells displayed on the scattergram. It is a very useful feature for the cytometer to provide both absolute counts of numbers of each cell type found per cubic millimeter, as well as percentages  
20 of each cell type relative to total cells measured. In order to provide such absolute counts and percentages, the counts are based on measurement of a known specimen volume. Thus, an initial specimen is precisely diluted prior to placement on the loading vehicle and the cytometer determines the volume of each testing  
25 chamber to obtain precise volumetric count. In order for the cytometer to determine the chamber volume (completely filled by the specimen), the individual chamber depth (distance from substrate to coverslip or coverglass is made with a known fixed thickness). The laser scanning cytometer is capable of scanning  
30 over a fixed known area by fixing the extent of the laser scan that is digitized and keeping track of the number of known step size steps that are moved in the perpendicular direction to complete the assay. Since the area scanned, the specimen dilution factor, and the loading vehicle chamber thickness or depth are  
35 known, the assay volume can be calculated, with the absolute count being thereby related to the undiluted absolute count, by the dilution factor.

Alternatively, the cytometer itself is modified to measure the depth of the chamber prior to scanning the specimen. A manner of effecting such measurement is by automatically focussing on indicia printed on the two inner surfaces which define the chamber depth and measuring the distance between the focus points.

#### DETAILED DESCRIPTION OF THE DRAWINGS AND THE PREFERRED EMBODIMENT

Figure 1 is a diagram of the Loading Vehicle (LV) 10 (shown as a microscope slide) made by applying double stick tape to a standard microscope slide in the areas 1, as spacer elements. Application of the tape in this pattern creates a set of horizontal chambers 2a-f, each connected to a specimen insertion trough (specimen reservoir) 3 and an exit trough 4. For effective use, four  $\mu$ l of a mixture of two antibodies are added to each horizontal chamber, which antibodies are specific to proteins on the surface or within human white blood cells. As described in the subsequent example herein, One of these antibodies CD19, conjugated to the dye phycoerythrin (PE), is used in all chambers (five of the chambers are described as being actually used in the example). All of the other antibodies are conjugated to the dye fluorescein isothiocyanate (FITC). From the bottom chamber 2a, the second antibody added to each successive chamber (2a-2e) is specific for CD3, CD5, CD10, Kappa and Lambda. In use, the antibodies are allowed to dry to a gel at which time a cover slip glass 5 (more clearly seen in Figure 2) is applied over the double stick tape.

Figure 3 shows an alternative loading vehicle 10' with imbedded chambers 2a'-f', in which the chambers are formed within the substrate rather than with spacer tape 1 as in Figures 1-2.

After data acquisition, the cells are stained within the single loading device of Figures 1 and 2, for microscopic examination. Approximately 500  $\mu$ l of the desired staining solutions are placed in succession into the trough in the loading vehicle and allowing them to drain through by gravity or by being drawn into absorbent material at the exit end of the capillary chamber. More than 90% of the cells are retained within the loading device, in part aided

by the fact that most staining procedures for light microscopy begin with an organic solvent fixation. Thus, one single specimen can be used for immunophenotyping and subsequent microscopic examination making full use of the LSC's capability to relocalize for visualization only cells that meet particular user-defined criteria of fluorescence, light scatter, size, etc.

The following examples illustrate the simultaneous testing regimen of the present invention as compared to the individual testing of the same specimens. It is understood that such examples are for illustrative purposes only and that details contained therein are not to be construed as limitations on the present invention.

#### EXAMPLE 1

In a testing regimen, the testing vehicle of partitioned microscope slide 10 of Figures 1 and 2 is utilized, with 240  $\mu$ l of a phosphate buffered saline suspension of human benign lymphoid cells from a hyperplastic tonsil being pipetted into specimen trough 3 therein. After 15 minutes, phosphate buffered saline is added to the insertion trough and an exit trough 4 is wicked, in order to wash the cells now adhered to the microscope slide.

The slide is placed on the stage of an LSC™ (laser scanning cytometer from CompuCyte Corp.) scanning device and the protocol of the device is set to make it scan a pattern consisting of a set of single strips each centered along each horizontal chamber. The LSC device images a 5 micron diameter laser beam on the slide surface in a scan pattern which consists of a series of vertical lines approximately 300 microns in length along a strip which, in this case, is 50 mm long. As each cell is encountered by the laser beam both scatter and fluorescence emissions from dyes in the cell are detected by sensors. The LSC is set to measure three properties of each cell encountered by the laser beam, forward angle light scatter (FALS) used to detect the presence of and contour or delineate each cell, green fluorescence to measure FITC, and red fluorescence to measure PE.

Results of the run of the specimen are shown in Figures 5a-g.

Figure 5a provides the area versus FITC peak for all cells used to gate single cells. Figure 5b is a map of the loading vehicle slide showing the cells in each lane or chamber. Figures 5c-g are a series of scattergrams which display dots representing each cell measured, the coordinates of which are proportional to each of two measurements. The first scattergram (Figure 5a) of the area of each cell as measured using FALS and peak FITC fluorescence is used to isolate single cell events from multiple cell events and debris. Only cells which have coordinates within the single cell gate area are presented as input to the other displays. The second scattergram (Figure 5b), in which the X and Y position of each cell is plotted, is used to gate all of the cells measured into five different scattergrams (Figures 5c-g), each one of which now obtains data from a single different scan strip corresponding to each horizontal chamber of the vehicle of Figure 1. Each of the scattergrams (5c-g), which use logarithmic scales, show a plot of each cell's FITC versus PE fluorescence resulting from the antibody reactions in its corresponding chamber (CD19 in each chamber with CD3, CD5, CD10, Kappa and Lambda in each of Figures 5c-g, respectively). Each of these scattergrams are divided into four quadrants. The number of cells in each quadrant can be tabulated to obtain a numerical value for the number of cells positive or negative for each combination of two antibodies. These values are printable on a report, thereby providing useful diagnostic information to a clinician.

In comparison to the results of the above tests, the following Example illustrates results obtained with prior art individual testing methods, wherein the results are essentially indistinguishable from that of the present invention.

## EXAMPLE 2

A portion of a specimen from the same source as that used in Example 1 is divided into aliquots in microtiter tubes, with each aliquot being reacted with a different pair of fluorochrome-labelled antibodies (the same pairs as in Example 1). The cells are then washed by centrifugation and a drop from each microtiter tube is placed on each of separate slides, with data

being acquired with the LSC scanning device for each slide aliquot individually.

Scattergrams (Figures 6a-g) are generated individually using each specimen placed onto a single microscope slide, as described  
5 above, after reacting with the five antibody pairs. The five sets of scattergrams from Example 1 and Example 2 are substantially identical.

It is understood that there are many other means of reacting  
10 affixed cells with immunofluorescent reagents in ways that create geographic partitioning. Thus, for example, affixation of the cells can be done through the use of a cytospin apparatus. Thereafter, different geographic regions of this layer of cells are reacted with multiple different mixtures of  
15 fluorochrome-labelled antibodies. The means of applying the antibodies in the correct geographic locations may vary from complex multi-channel fluidics systems to manual pipetting to systems utilizing a porous or sponge-like material possibly pre-impregnated with the antibody mixtures. The use of pre-  
20 impregnated pads or some other potentially disposable means of delivering the antibody pairs or groups to a layer of affixed cells is preferred in order to eliminate time consuming and technically demanding pipetting, as well as to automatically create geographic checkerboards of different antibody mixtures. As with the preloaded devices described above, such pre-  
25 impregnated pads may be designed to contain antibody panels specific for different clinical differential diagnoses, thereby reducing waste for hospital laboratories.

What is claimed is:

1. A stationary testing media device adapted for the conducting of multiple simultaneous chemical reaction tests on a single testing sample, said device comprising a substrate partitioned into at least two testing zones, with each of the zones having been pre-loaded with a stable test reactant, wherein the testing zones are physically separated from one another, the device further comprising a single test sample reservoir connected to each of the testing zones with a fluid flow connection means, which fluid flow connection means simultaneously feeds each of the testing zones with a test portion from the test sample in said reservoir, whereby at least two of the test portions separately react with stable reactant in at least two of the testing zones to provide separate test results.

2. The device of claim 1 wherein said substrate is one of a glass or plastic plate.

3. The device of claim 2, wherein the test sample reservoir comprises a trough and said zones comprise individual troughs, each of which is connected by fluid connection to the reservoir trough.

4. The device of claim 3, wherein the reservoir trough and zone troughs are situated below a surface of the plate.

5. The device of claim 3, wherein the reservoir trough and zone troughs are situated above a surface of the plate.

6. The device of claim 3, wherein the zone troughs each has two ends with a first end being in fluid connection with the reservoir trough and wherein the second end is connected by fluid connection with a drain trough.

7. The device of claim 6, wherein the plate has a length and width and wherein the reservoir trough extends along said width and said zone troughs extend along said length in separated parallel juxtaposition with each other.

8. The device of claim 3, wherein said test sample comprises cells and wherein the zone troughs retain sufficient cells therein for reaction with the reactants contained within the individual troughs whereby the reaction product is scannable by a laser scanning cytometer.

9. The device of claim 7, wherein the stable reactants comprise cell staining material.



10. The device of claim 1, wherein depth of each of the individual zones is of predetermined dimensions.

11. The device of claim 1, wherein the device comprises means for permitting external determination of depth of each of the individual zones.

12. The device of claim 1, wherein said substrate is a microscope slide.

13. A method of conducting at least two simultaneous tests with a laser scanning cytometer, with a single test sample on a single glass or plastic plate, said method comprising the steps of:

a. providing the plate with a test sample reservoir and at least two separated sample portion troughs in fluid connection with the reservoir;

b. providing each of the sample troughs with a stable reactant for a test sample;

c. placing a fluid test sample in said reservoir wherein fluid connection means causes portions of the test sample to flow into each of the at least two separated sample portion troughs;

d. maintaining sufficient cells in each of the portions in said portion troughs for a time sufficient for the cells to react with the stable reactant within the individual portion troughs; and

e. removing excess reactant cells whereby test results can be meaningfully obtained by the laser scanning cytometer with minimized background noise.

14. The method of claim 13, wherein depth of each of the sample troughs is of predetermined dimensions and wherein the method further comprises the steps of:

i) precisely diluting the fluid test sample prior to placement in said reservoir;

ii) scanning the individual fluid test samples with the laser scanning cytometer over a fixed known area by fixing the extent of the laser scan that is digitized and keeping track of the number of known step size steps that are moved in a perpendicular direction to complete an assay;

iii) calculating the assay volume; and

iv) thereby determining an absolute count and an undiluted absolute count.

15. The method of claim 13, wherein the method further comprises the steps of:

i) precisely diluting the fluid test sample prior to placement in said reservoir;

5 ii) determining the depth of each of the sample troughs;  
iii) scanning the individual fluid test samples with the laser scanning cytometer over a fixed known area by fixing the extent of the laser scan that is digitized and keeping track of the number of known step size steps that are moved in a perpendicular direction to complete an assay;

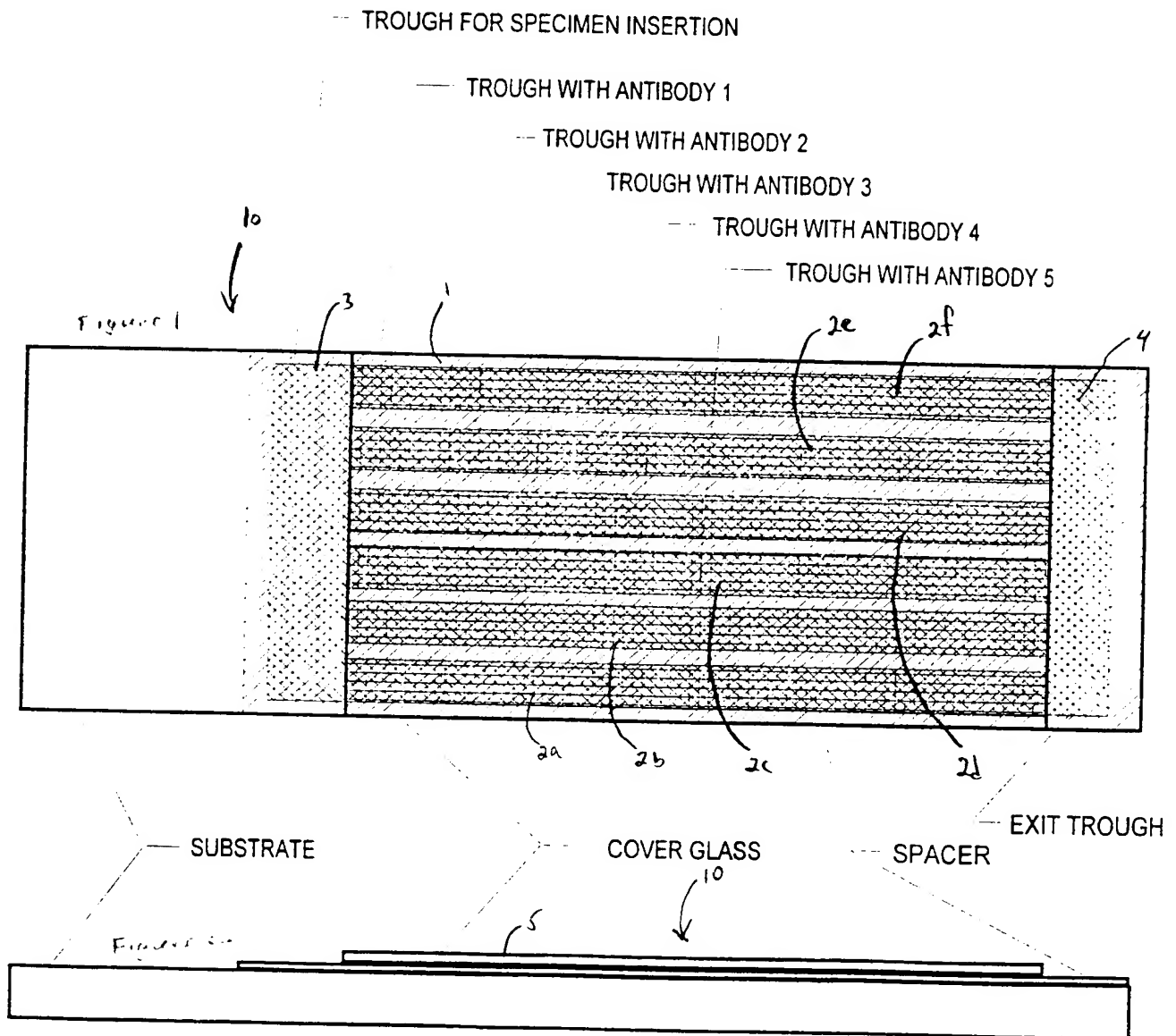
10 iv) calculating the assay volume; and

v) thereby determining an absolute count and an undiluted absolute count.

16. The method of claim 15, wherein the depth of each trough is determined by separately focussing a laser of the laser scanning cytometer on indicia printed on two inner surfaces which define the depth of the trough and measuring the distance between focus points so obtained.

17. A stationary testing media device adapted for the conducting of multiple simultaneous chemical reaction tests on a single testing sample, said device comprising a substrate partitioned into at least two testing zones, wherein the testing zones are physically separated from one another, the device further comprising a single test sample reservoir connected to each of the testing zones with a fluid flow connection means, which fluid flow connection means simultaneously feeds each of the testing zones with a test portion from the test sample in said reservoir, whereby at least two of the test portions separately react with reactant materials introduced into the separate test portion, in at least two of the testing zones, to provide separate test results.

1/4



2/4

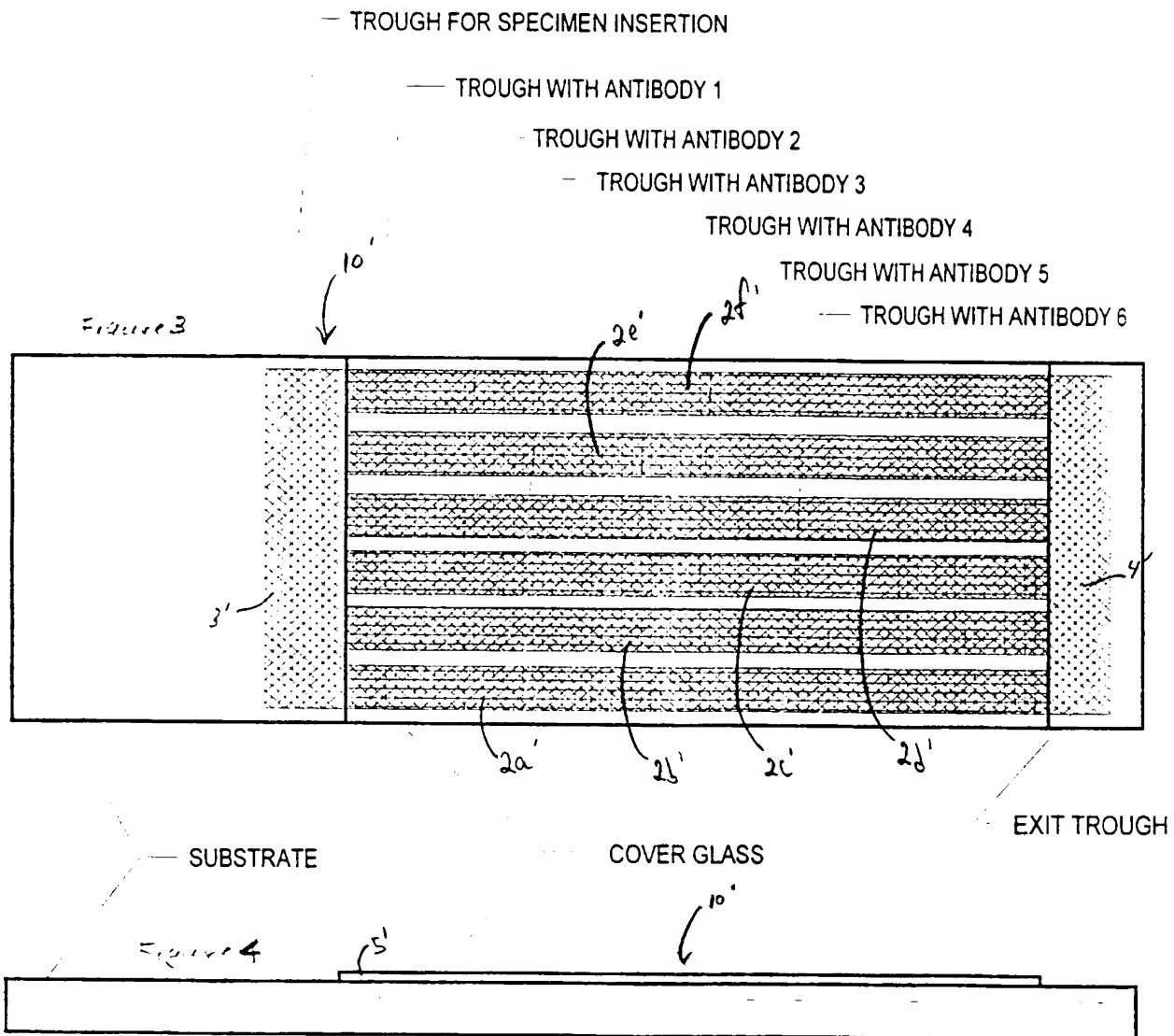


Figure E

3/4

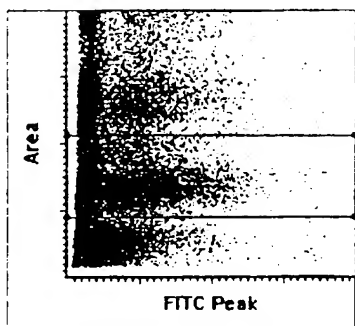


Figure 5a

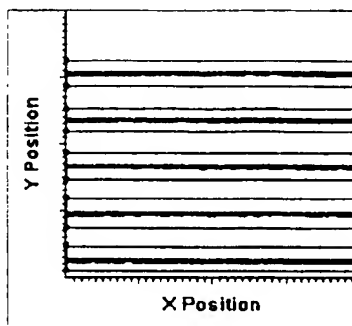


Figure 5b

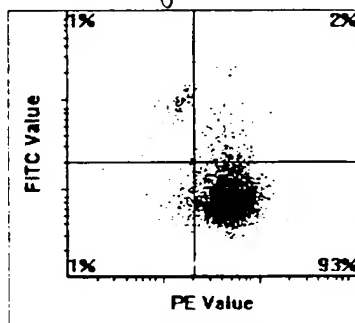


Figure 5c

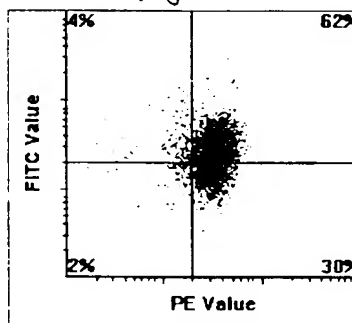


Figure 5d

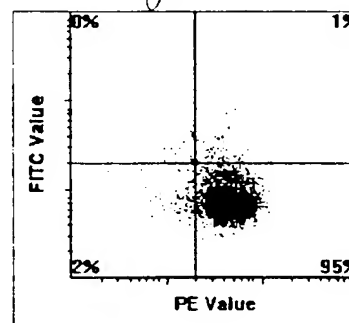


Figure 5e

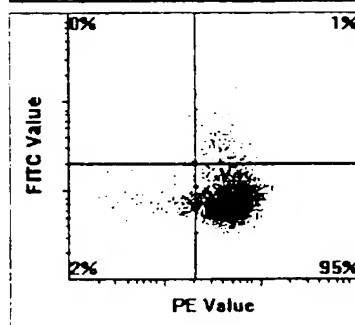


Figure 5f

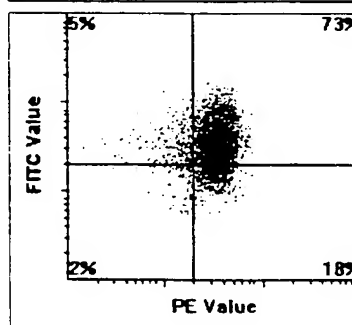
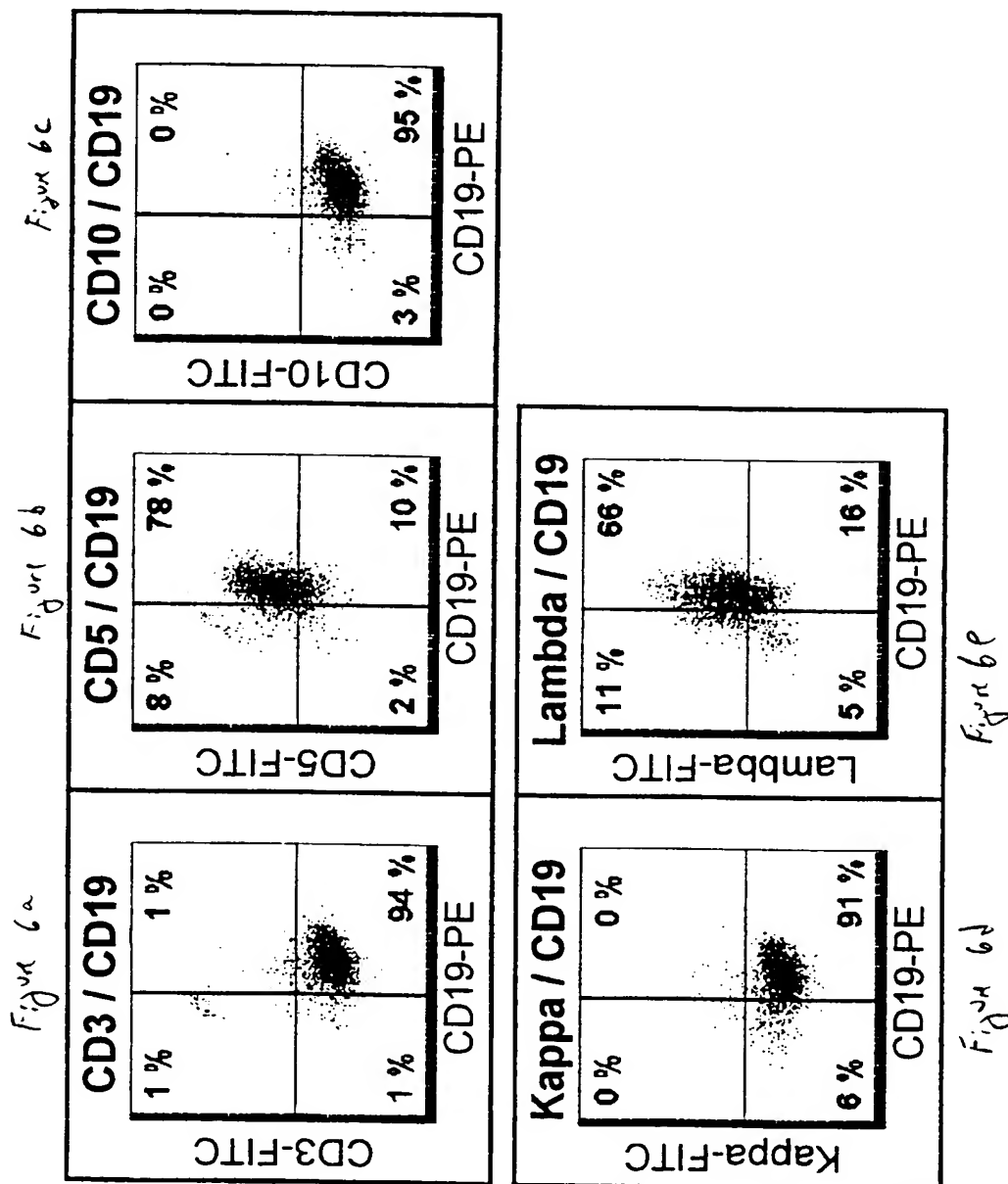


Figure 5g

Figure 6



# INTERNATIONAL SEARCH REPORT

International application No.

PS 97/03900

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,296,375 A (KRICKA et al.) 22 March 1994, column 3, lines 65-68, column 4, lines 1-26, column 10, lines 24-38 and Figure 9.	1-3, 5, 10 ----- 6- 9, 11-16
X --- Y	US 3,799,742 A (COLEMAN) 26 March 1974, column 11, lines 50-75, column 12, lines 1-9, column 16, lines 50-75 and Figures 18 and 29.	1-4, 17 ----- 6-9, 11-16
Y	US 4,963,498 A (HILLMAN et al.) 16 October 1990, column 19, lines 37-68 and Figure 2A.	6-7
Y	US 5,072,382 A (KAMENTSKY) 10 December 1991, column 1, lines 54-68, columns 2-5 and column 7, lines 17-68.	8-9 and 11-16

☐

Further documents are listed in the continuation of Box C.

☐

See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

18 APRIL 1997

Date of mailing of the international search report

12 MAY 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MAUREEN WALLENHORST

Telephone No. (703) 308-0651

Form PCT/ISA/210 (second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.  
US97/03900

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

G01N 33/48, 21/00, 21/01, 21/03, 1/10; B01L 3/00

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

436/63, 165; 422/58, 82.05, 99, 102; 356/244, 246; 435/287.9, 288.3, 288.4, 288.7, 305.1, 305.2

## B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

436/63, 164, 165; 422/55, 57, 58, 68.1, 73, 82.05, 82.08, 99, 102; 356/244, 246; 435/7.1, 7.2, 7.21, 7.23, 7.24, 287.2, 287.9, 288.3, 288.4, 288.7, 305.1, 305.2, 307.1; 250/432R, 461.2